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Antiproliferative prostaglandins activate heat shock transcription factor

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ABSTRACT **Treatment of human K562 erythroleukemia cells with the antiproliferative prostaglandin A_1 results in the elevated transcription of two heat shock genes, *HSP70* and *HSP90*. Parallel with increased heat shock gene transcription is the activation of heat shock transcription factor. Heat shock transcription factor levels are induced within 60 min after prostaglandin A_1 addition to levels similar to that achieved during heat shock. The requirement for protein synthesis for prostaglandin A_1 activation of heat shock transcription factor suggests that effects on nascent protein synthesis may be involved in the signaling mechanism. Although it is unclear whether the activation of a heat shock response by prostaglandins is relevant to the biochemical properties of these natural substances, cells pretreated with prostaglandin A_1 are protected against a subsequent heat shock, indicative of a thermotolerant state.**

Prostaglandins are a class of naturally occurring cyclic 20-carbon fatty acids that are synthesized from polyunsaturated fatty acid precursors in response to external stimuli such as cell injury and inflammation (1). Prostaglandins function as intracellular hormones involved in the regulation of various physiological and pathological processes of eukaryotes, including cell proliferation and differentiation (2), the immune response (3), inflammation (4), cytoprotection (5, 6), and the febrile response (7).

The type A and J prostaglandins, characterized by the presence of a reactive α,β-unsaturated ketone in the cyclopentane ring (cyclopentenone prostaglandins), have antiproliferative activity and cause cultured mammalian cells to arrest in the G_1 phase of the cell cycle (8–10). Human erythroleukemia K562 cells, for example, are extremely sensitive to prostaglandin A_1 (PGA_1), which results in nearly complete cessation of cell growth at doses that do not affect cell viability and do not suppress DNA or RNA synthesis for at least 24 h (11, 12). Treatment with PGA_1, PGA_2, and PGJ_2 results in the elevated synthesis of HSP70, a major heat shock and stress-induced protein (12, 13). Because HSP70 expression is also growth regulated (14, 15), we reasoned that induction of HSP70 by prostaglandins could represent a response to the growth-related effects of this compound. The growth-regulated response of the human *HSP70* gene requires cis-acting elements in the basal promoter that are distinct from the distally located heat shock elements on the heat shock gene promoter, which are necessary for heat shock and other forms of stress responsiveness. The cellular response to a wide range of external stress stimuli, including heat shock, heavy metals, amino acid analogues, oxidizing agents, and teratogens (16), involves the activation of heat shock transcription factor (HSF), which binds to the heat shock element (HSE), comprised of multiple adjacent inverted repeats of the pentamer *n*GAA*n* (17, 18).

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In this study, we show that the cyclopentenone prostaglandin PGA_1 induces in human cells the transcription of classical heat shock genes through activation of HSF. The activation of HSF by PGA_1 requires continued protein synthesis, thus suggesting a role for PGA_1 in the modification of nascent proteins.

MATERIALS AND METHODS

Cell Culture. K562 cells were grown at 37°C in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum at densities of 1×10^5 to 1×10^6 cells per ml. Prostaglandins (Cayman Chemicals, Ann Arbor, MI), stored as 100% ethanol stock solution (10 mg/ml), were used at a concentration of 4 μg/ml, unless differently specified, and control cells received a corresponding volume of ethanol.

Transcription, mRNA Levels, Protein Synthesis, and Gel Mobility Shift Assays. *In vitro* run-on transcription reactions were performed in isolated K562 nuclei as described (19). ^{32}P-labeled RNA was hybridized to nitrocellulose filters containing plasmids for the following human genes, *HSP70* (pH2.3; ref. 20), *HSP90* (pUCHS801; ref. 21), *HSP60* (pUC601; ref. 22), *GRP78/BiP* (pHG23.1; ref. 23), *p72/HSC70* (pHA7.6), β-actin (24), and c-*myc* (pSV-TC; ref. 25), and vector (pGEM2; Promega). Following hybridization, filters were visualized by autoradiography and the radioactivity was quantitated by a Molecular Dynamics Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

Total cytoplasmic RNA was isolated (26), fractionated on 1% agarose/formaldehyde gels, transferred to nitrocellulose, and hybridized with nick-translated ^{32}P-labeled *HSP70* gene sequences.

Cells were metabolically labeled with [^{35}S]methionine for 45 min and equal amounts of protein were analyzed on 10% SDS/PAGE gels. For immunoblot analysis, the nitrocellulose filters were incubated with monoclonal antibody 3A3, which recognizes HSP70 and p72/HSC70 (S. P. Murphy, personal communication). The filters were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Boehringer Mannheim), and specific complexes with HSP70 and p72/HSC70 were detected by enhanced chemiluminescence (Amersham).

Whole cell extracts were prepared and binding reactions were performed using a specific heat shock element probe as described (27). HSF–HSE complexes were analyzed by nondenaturing 4% polyacrylamide gel electrophoresis.

RESULTS

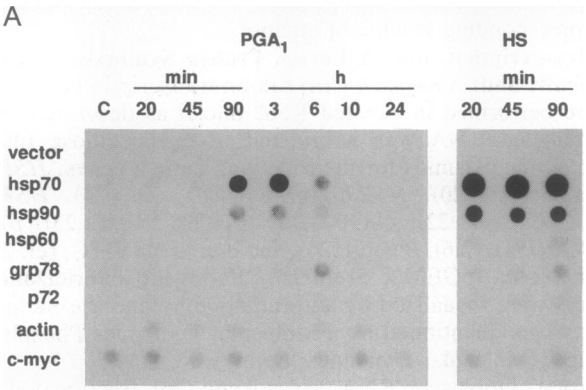
PGA_1 Induces Transcription of *HSP90* and *HSP70*. In previous studies it was observed that treatment of cultured human cells with PGA_1, PGE, and PGF resulted in the elevated synthesis of HSP70 only in cells treated with PGA_1. To examine whether PGA_1 exerted its effect at the transcriptional level, we measured the effects of PGA_1 on the tran-

Abbreviations: PG, prostaglandin; HSF, heat shock transcription factor; HSE, heat shock element.

scription of a number of human genes, including the heat shock genes *HSP90*, *HSP70*, and *HSP60*, *GRP78/BiP* and *p72/HSC70*, as well as two control (nonstress) genes, β-actin and c-*myc*. Knowledge of which genes were induced by PGA_1 would reveal whether the effects of PGA_1 were specific.

To measure the effects of PGA_1 on transcriptional activation, we performed *in vitro* nuclear run-on assays with nuclei isolated from K562 cells at different times during PGA_1 treatment. The transcription rate of the *HSP70* gene increased rapidly between 45 and 90 min, attained a maximal 15-fold induction within 3 h, and thereafter declined to basal levels by 10 h (Fig. 1). By comparison, heat shock induced *HSP70* transcription by 50-fold (Fig. 1*A*, HS). Transcription of *HSP90*, though less striking, exhibited similar kinetics to *HSP70*. In contrast to the relatively rapid induction of *HSP70* and *HSP90*, *GRP78/BiP* transcription was consistently induced 4-fold only at 6 h of PGA_1 treatment. The transcription of another heat shock gene (*HSP60*), the constitutively expressed *p72/HSC70*, and the control β-actin and c-*myc* genes was unaffected. These results clearly demonstrate that PGA_1 acts at the transcriptional level and preferentially induces two classical heat shock genes, *HSP70* and *HSP90*.

We examined the effects of PGA_1 on HSP70 mRNA levels during PGA_1 treatment. Cytoplasmic RNA was isolated at different times following PGA_1 treatment and HSP70 mRNA levels were measured by Northern blot analysis (Fig. 2*A*).



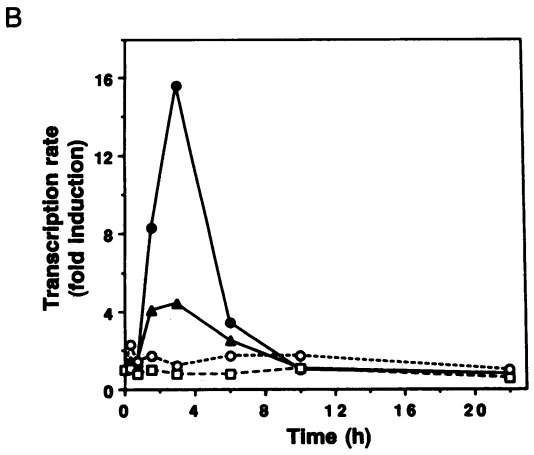


FIG. 1. Effects of PGA_1 treatment on the transcription of control (nonstress) and heat shock genes in K562 cells. (*A*) Autoradiogram of the relative transcription rates of plasmid vector (pGEM2), *HSP70*, *HSP90*, *HSP60*, *GRP78*, *p72/HSC70*, β-actin, and c-*myc* genes at the indicated times (min, h) during PGA_1 treatment or following heat shock (at 42°C) for periods of 20, 45, and 90 min. (*B*) Quantitative analysis using a Molecular Dynamics Phosphorimager analyzer of *HSP70* (●), *HSP90* (▲), β-actin (□), and c-*myc* (○) transcription rates shown in *A*. The values are expressed as arbitrary units obtained by comparing transcription rates to control levels.

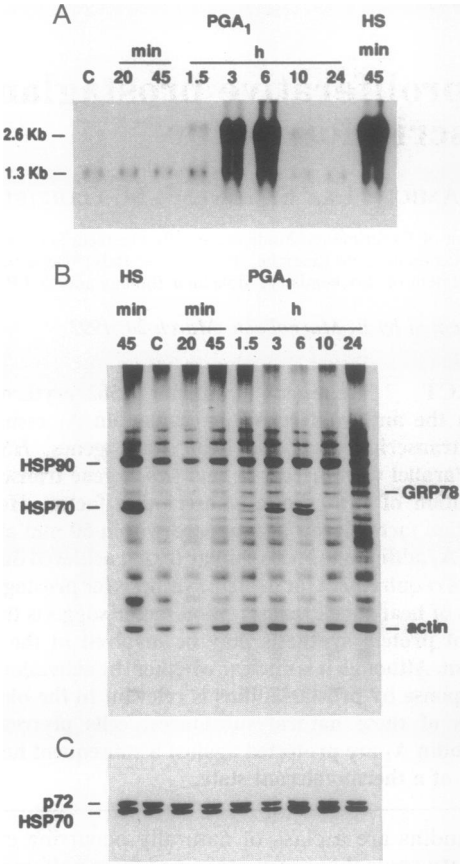


FIG. 2. Effect of PGA_1 treatment on HSP70 mRNA levels and HSP70 protein synthesis and accumulation. (*A*) Northern blot analysis of cytoplasmic RNA isolated at various times of PGA_1 treatment on control (C) or heat-shocked (HS) cells. The band at 2.6 kilobases corresponds to the hybridization with the HSP70 probe and the 1.3-kb band corresponds to the rat glyceraldehyde phosphate dehydrogenase mRNA used as an internal control for the loading of RNA. (*B*) Autoradiography of SDS/PAGE analysis of [^{35}S]methionine-labeled proteins in control, heat-shocked (HS), or PGA_1-treated K562 cells. The positions of HSP90, HSP70, GRP78, and actin are indicated. (*C*) Western blot analysis of duplicate samples as indicated in *B*, incubated with the monoclonal antibody 3A3, which recognizes p72/HSC70 and HSP70 proteins.

HSP70 mRNA levels were elevated slightly at 90 min, with maximal HSP70 mRNA levels detected at 3–6 h. Thus, the increase in HSP70 mRNA levels followed the increase in *HSP70* transcription rates (compare Fig. 1*A* to Fig. 2*A*). The effects of PGA_1 on the overall levels and patterns of protein synthesis, specifically HSP70 synthesis, were examined by pulse-labeling with [^{35}S]methionine and SDS/PAGE. Slightly increased levels of HSP70 synthesis were detected by 90 min of PGA_1 treatment, coincident with the initial increase in HSP70 mRNA levels, and reached higher levels of synthesis between 3 and 6 h (Fig. 2*B*). HSP90 synthesis was induced slightly after 90 min of PGA_1 treatment and GRP78/BiP synthesis was elevated only at later time points. Synthesis of other stress-responsive proteins and overall levels of protein synthesis were not affected by PGA_1 treatment. The results of Western blot analysis using a monoclonal antibody (3A3) that recognizes p72/HSC70 and HSP70 revealed that HSP70 levels gradually accumulated during PGA_1 treatment to a 3-fold higher level by 6–10 h (Fig. 2*C*). Although the PGA_1 induction of *HSP70* gene expression was transient, high levels of HSP70 persisted for periods up to 24 hours. The effects of PGA_1 appear to be specific to HSP70 since the levels of p72/HSC70 were unaltered.

PGA_1 Treatment Activates HSF. Two lines of evidence suggested that PGA_1 induced *HSP70* transcription through activation of HSF. First, the kinetics of induction were rapid, and second, two heat shock-induced genes, *HSP70* and *HSP90*, were induced. These results suggest that transcriptional activation is mediated by HSF. To demonstrate whether PGA_1 induced HSF, we used the gel mobility shift assay using a synthetic oligonucleotide containing the consensus HSE binding site from the human *HSP70* promoter. Gel mobility shift assays were performed with whole cell extracts prepared from cells treated with the same dose (4 μg/ml) of PGA_1, PGE_2, and PGF_1. Only PGA_1 treatment induced the appearance of a HSF–HSE complex, which was indistinguishable from the heat shock-induced HSF–HSE complex (Fig. 3*A*). HSF–HSE complex formation was detected within 90 min of PGA_1 treatment, attained maximal levels between 3–6 h, and declined after 10 h. Kinetics of HSF DNA-binding activity induced by PGA_1 and heat shock are presented schematically in Fig. 3*B*, showing a pattern that closely parallels the induction of *HSP70* transcription (Fig. 1*A*). It was of interest to note that the level of *HSP70* transcription was significantly lower in PGA_1 versus heat-shocked cells even though the levels of HSF were equivalent in cells subjected to either treatment (compare Fig. 1*A* to Fig. 3 *A* and *B*). To further characterize the PGA_1-induced activation of HSF and heat shock gene transcription, we performed a dose–response experiment in which K562 cells were treated for 3 h with different concentrations of PGA_1. The HSF DNA-binding activity and the *HSP70* transcription were induced when cells were treated with PGA_1 at concentrations >2 μg/ml (data not shown).

Activation of HSF by PGA_1 Requires Nascent Protein Synthesis. Little is known about the mechanism of action of the cyclopentenone prostaglandins. It has been reported that PGA_1 is rapidly incorporated into cells and binds to nuclear proteins and DNA (28). One possibility is that PGA_1 interacts with, modifies, and alters the conformation of certain proteins, thus activating the heat shock transcriptional response. To test this hypothesis, we examined whether the PGA_1 induction of heat shock gene transcription and HSF activation was dependent on *de novo* protein synthesis. Cells were treated with 100 μg of cycloheximide per ml for 30 min prior to PGA_1 administration. Cells were removed at various times following treatment and assayed for HSF DNA-binding and transcriptional activities. As shown in Fig. 4 *A* and *C*, cycloheximide pretreatment abolished the ability of PGA_1 to induce *HSP70* and *HSP90* transcription and activation of HSF. These results strongly suggest that *de novo* protein synthesis is necessary for PGA_1-induced activation of the heat shock response.

The requirement for protein synthesis for activation of HSF during treatment with PGA_1 contrasts with the requirements for protein synthesis in activation of the heat shock response. Cells were pretreated with cycloheximide and subsequently incubated at 37°C, 42°C, 43°C, and 45°C. As shown in Fig. 4*B*, *de novo* protein synthesis is required for activation of HSF and heat shock gene transcription at 42°C but not at 43°C and 45°C. These results demonstrate that activation of a 42°C heat shock response is dependent on protein synthesis, which suggests that the mechanism of 42°C heat shock involves damage to nascent translation products. In addition, we suggest that heat shock at 43°C or 45°C affects

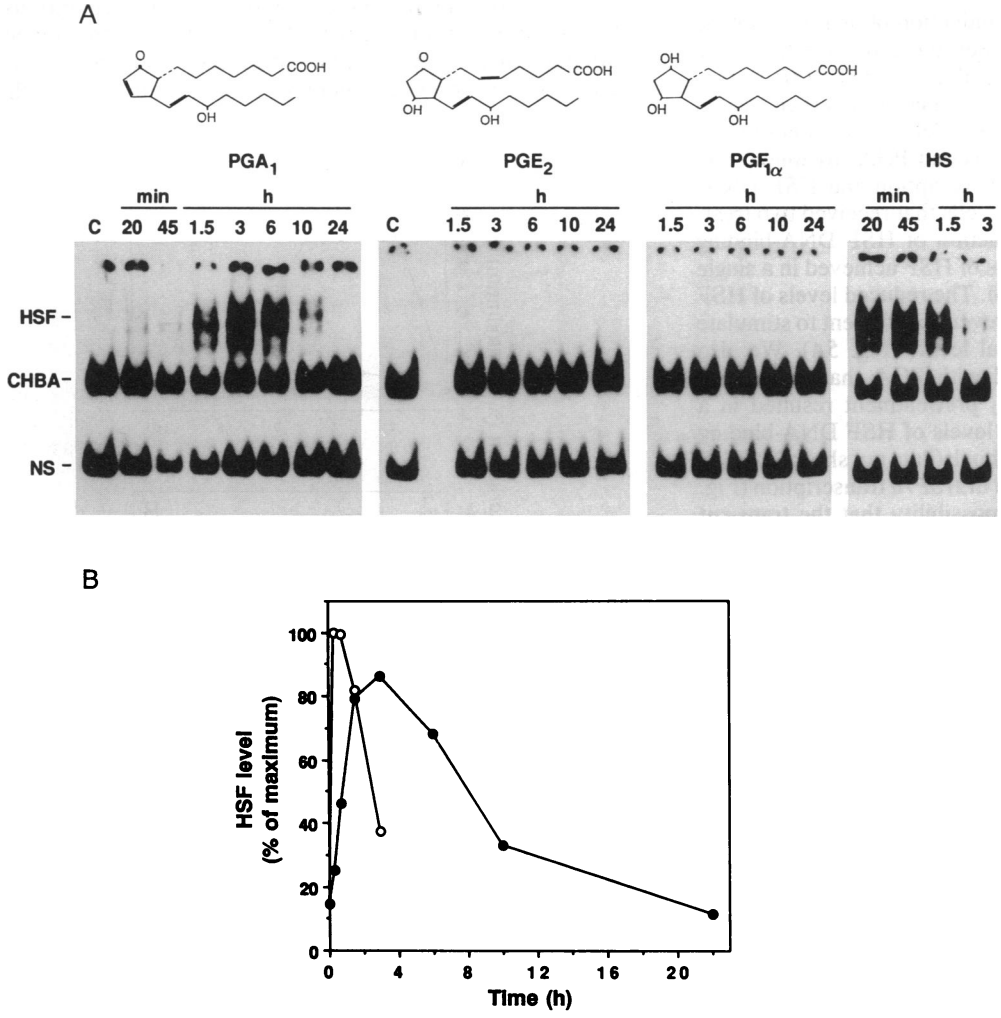


FIG. 3. Selective induction of HSF DNA-binding activity by PGA_1. (*A*) K562 cells were treated with PGA_1, PGE_2, and $PGF_{1\alpha}$ (formula as shown above each corresponding panel) at 4 μg/ml for indicated time periods, and whole cell extracts were prepared for gel mobility shift assay using the HSE sequence from the human *HSP70* promoter (27). The panel with heat shock (HS) indicates the HSF DNA-binding activity of K562 cells subjected to 42°C for indicated time periods. HSF denotes the induced form of DNA-binding activity, CHBA indicates the constitutive HSE-binding activity (27), and NS denotes nonspecific protein–DNA interactions. (*B*) The levels of HSF DNA-binding activity in PGA_1-treated (closed circles) and heat-shocked (open circles) cells were quantitated with a Molecular Dynamics Phosphorimager. HSF values were corrected against the nonspecific (NS) signal and normalized to the level of HSF DNA-binding activity at 45 min of heat shock, which was given a value of 100%.

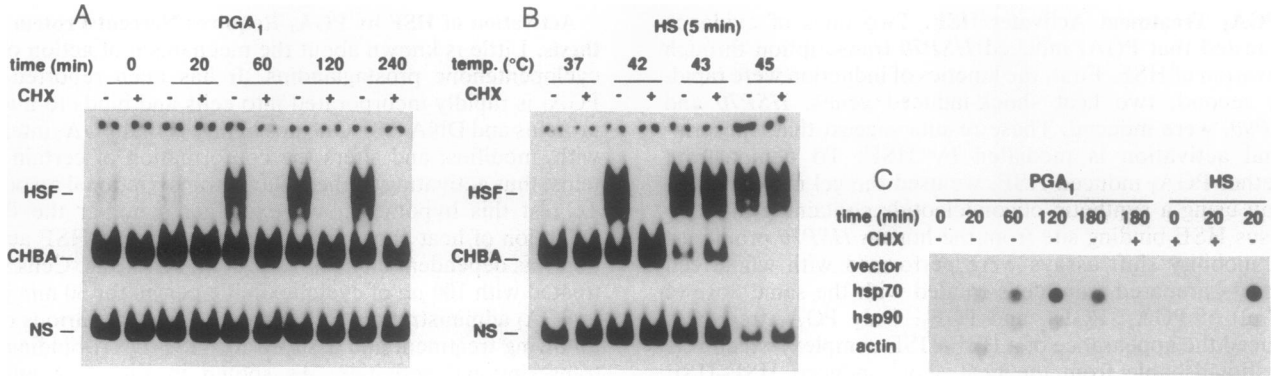


FIG. 4. Effect of cycloheximide treatment on PGA_1 activation of HSF and heat shock gene transcription. (*A*) K562 cells were untreated (−) or treated (+) for 30 min with cycloheximide (100 μg/ml) prior to PGA_1 treatment for periods as indicated. Whole cell extracts were prepared for gel mobility shift assay using the HSE probe. (*B*) K562 cells were untreated (−) or treated (+) with cycloheximide prior to a 5-min heat shock at 42°C, 43°C, and 45°C. (*C*) Transcription rates of the *HSP70*, *HSP90*, and β-actin genes measured by nuclear run-on assay from the same samples as shown in *A* and *B* (HS at 42°C).

nascent and preexisting proteins, thus providing the signal for activation of the heat shock response that is independent of protein synthesis.

Exposure to Prostaglandin Attenuates Subsequent Prostaglandin Treatment or Heat Shock Response. During the continuous exposure of cells to PGA_1, the transcriptional induction of *HSP70* and activation of HSF represent a transient response. This can be explained by the depletion of PGA_1 from the culture medium, reducing the effective concentration, even though PGA_1 has been shown to be stable over a 24-h period at 37°C (29). An alternative possibility is that cells continuously exposed to PGA_1 become adapted to PGA_1, perhaps related to the transient induction of heat shock gene expression. To distinguish between these possibilities, cells were treated for 15 h with PGA_1, during which *HSP70* transcription rates were induced and attenuated, and then treated with an additional aliquot of PGA_1. Cells were withdrawn at different time points during the second PGA_1 treatment and the levels of heat shock gene transcription and HSF DNA-binding activity were measured. Cells that received two treatments of PGA_1 had a 50% reduction of HSF DNA-binding activity as compared to the levels of HSF achieved in a single PGA_1 treatment (data not shown). The reduced levels of HSF DNA-binding activity were, however, insufficient to stimulate *HSP70* transcription above basal levels (Fig. 5*A*). We also examined whether cells treated with PGA_1 had an altered sensitivity to heat shock. PGA_1 pretreatment resulted in a consistent 20% reduction in the levels of HSF DNA-binding activity, induced by a 42°C heat shock (data not shown), which corresponded to a 60% reduction in *HSP70* transcription (Fig. 5*B*). These results exclude the possibility that the transient heat shock response during PGA_1 treatment is due to depletion of PGA_1 in the culture medium and suggest that PGA_1-treated cells acquire a form of "tolerance" to subsequent exposures to PGA_1 or heat shock.

DISCUSSION

The studies presented here demonstrate that PGA_1 treatment of human K562 cells has a significant effect on the expression of two major heat shock genes, *HSP70* and *HSP90*. The inducible transcription of both genes by PGA_1 is mediated by HSF, which is activated from its control non-DNA-binding state to a transcriptionally active form. Our studies reveal a surprising specificity of the cyclopentenone prostaglandins since only PGA_1 induces HSF DNA-binding activity and the closely related PGE and PGF do not induce the heat shock response.

The requirement of protein synthesis for PGA_1 induction of HSF activation suggests that PGA_1 may affect nascent proteins at concentrations that inhibit cell proliferation without altering cell viability (12). The effects of PGA_1 share features common to other inducers of the heat shock response, including heat shock, amino acid analogues, and the nitrosourea anti-cancer compounds (27, 30). The requirement for protein synthesis for induction of a heat shock response by these conditions is consistent with the widely held hypothesis that the heat shock response is induced by compounds or conditions that perturb protein synthesis. The coinduction of *GRP78/BiP* in addition to *HSP70* and *HSP90* by PGA_1, amino acid analogues, transition heavy metals, and heat shock reveals that PGA_1, as for the other conditions, also interferes with nascent protein synthesis in the lumen. Although there is very little information on the *in vivo* consequences of PGA_1, there is evidence to support the

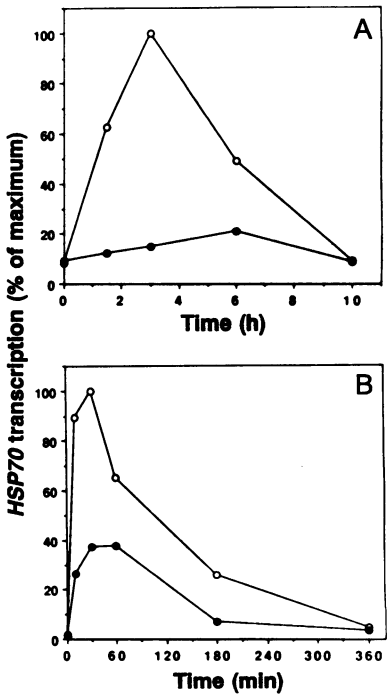


FIG. 5. Effect of PGA_1 pretreatment on the subsequent stress induction of heat shock genes. (*A*) Kinetics of *HSP70* transcription rates in cells treated with PGA_1 (4 μg/ml) for periods of 0–10 h (open circles) or after a 15-h pretreatment with PGA_1 (closed circles). (*B*) Kinetics of *HSP70* transcription rates in cells that were heat shocked for periods of 0–360 min at 42°C (open circles) or heat shocked for the same period after a 15-h pretreatment with PGA_1 (closed circles).

covalent interaction between PGA_1 and sulfhydryl groups of cysteine-rich proteins (31, 32). It will be of interest to examine the specificity of PGA_1 action in greater detail. Is the activation of HSF by PGA_1 due to direct effects on protein conformation? If PGA_1 interacts and modifies nascent proteins, it will be important to establish whether there are preferred or specific substrates. Because we have not been able to *in vitro* activate HSF by exogenous PGA_1 using HeLa cell S100 extracts, it seems unlikely that PGA_1 acts directly on the control non-DNA-binding form of HSF.

Even though these studies reveal a specific effect of the cyclopentenone prostaglandins on gene expression, an important question is whether the activation of a heat shock-like response by PGA_1 is biologically relevant to some of the known biological activities of PGA_1 as antiviral agents (23–29, 33–35) and in the antiproliferative response (10, 13, 36). The effects of prostaglandins and hemin on K562 cells share common features. Hemin treatment causes non-terminal erythroid differentiation, the activation of HSF DNA-binding activity, and the induction of *HSP70, HSP90*, and *GRP78/BiP* transcription (ref. 37; L.S. and R.I.M., unpublished data). Prostaglandins and hemin, unlike the traditional inducers of the heat shock response (e.g., inhibitors of energy metabolism, amino acid analogues, antineoplastic compounds), are natural substances. Whether the levels of the cyclopentenone prostaglandins achieve a sufficiently high local concentration *in vivo* to activate HSF and the transcription of the classical heat shock genes in affected cells or tissues has not been examined.

We have found that pretreatment with PGA_1, at concentrations that induce HSF DNA-binding activity, diminishes the magnitude of the subsequent stress response. The attenuation of the second exposure to stress, which occurs in PGA_1-pretreated cells, may be related to the accumulation of a 2- to 3-fold higher level of HSP70 protein. One explanation is that the induction and accumulation of high levels of HSP70 during the initial exposure to PGA_1 alters the ability of the treated cell to sense or respond to a subsequent stress stimulus and thus, by direct or indirect means, to regulate the activity of HSF. Consistent with this view, our laboratory has recently shown an interaction between HSP70 and HSF (38).

The role of PGA_1 in the regulation of the stress response is of particular interest as prostaglandins are a metabolic product of arachidonic acid, a central mediator of the inflammatory response. A possible link between the inflammatory response and the stress response can be suggested from two recent observations. Treatment of HeLa cells with salicylates and indocin, both nonsteroidal antiinflammatory drugs, as well as with arachidonic acid itself induces HSF DNA-binding activity (ref. 39; D. Jurivich, L.S., and R.I.M., unpublished data).

Note Added in Proof. Holbrook *et al.* (40) have recently made similar observations on PGA effects on the heat shock response.

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